Plant Signaling & Behavior 8:3, e23329; March 2013; © 2013 Landes Bioscience

Further insights into the isoenzyme composition and activity of glutamate dehydrogenase in *Arabidopsis thaliana*

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Keywords: *Arabidopsis*, glutamate dehydrogenase, isoenzymes, NADH-, NADPH-dependent activity, roots

Following the discovery that in *Arabidopsis*, a third isoenzyme of NADH-dependent glutamate dehydrogenase (GDH) is expressed in the mitochondria of the root companion cells, we have re-examined the GDH isoenzyme composition. By analyzing the NADH-GDH isoenzyme composition of single, double and triple mutants deficient in the expression of the three genes encoding the enzyme, we have found that the α, β and γ polypeptides that comprise the enzyme can be assembled into a complex combination of heterohexamers in roots. Moreover, we observed that when one or two of the three root isoenzymes were missing from the mutants, the remaining isoenzymes compensated for this deficiency. The significance of such complexity is discussed in relation to the metabolic and signaling function of the NADH-GDH enzyme. Although it has been shown that a fourth gene encoding a NADPH-dependent enzyme is present in *Arabidopsis*, we were not able to detect corresponding enzyme activity, even in the triple mutant totally lacking NADH-GDH activity.

In the last 25 years, good progress has been made in obtaining a better understanding of the role of alternative metabolic pathways in plants, which are potentially able to incorporate ammonium into organic molecules.[1] The reaction catalyzed by the mitochondrial enzyme NADH-dependent glutamate dehydrogenase (NADH-GDH, EC 1.4.1.2), has often been considered as being one of such alternative pathways, as the enzyme is able to incorporate ammonium into 2-oxoglutarate to form glutamate in vitro, or to function in the opposite direction to deaminate glutamate. Although it has been clearly demonstrated by the means of ^{15}N- and ^{13}C-labeling experiments that the latter deamination reaction occurs in plant cells, it has been regularly argued that under certain physiological conditions, when the ammonium concentration reaches a particular threshold, the enzyme is able to function in the aminating direction.[2-5] Consequently, the exact physiological function of the enzyme NADH-GDH was still matter of debate until Fontaine et al.,[6] demonstrated that in *Arabidopsis thaliana* three distinct nuclear genes each encode an active GDH protein. One named GDH3 is expressed only in the root companion cells, whereas the two others GDH1 and GDH2 are expressed in the same companion cells in both roots and shoots. The finding that a third NADH-GDH isoenzyme is active in *Arabidopsis* has demonstrated through the use of mutants deficient in the expression of the three NADH-GDH genes, that the main function of the enzyme is to provide 2-oxoglutatarate for the tricarboxylic acid cycle, primarily in the roots.[6] Thus, such a demonstration reinforced the current consensus that NADH-GDH plays virtually no role in the assimilation of inorganic nitrogen and that, whatever its metabolic origin, ammonia is mostly if not exclusively incorporated into organic molecules through the glutamine synthetase-glutamate synthase (GS-GOGAT) assimilatory pathway.[7,8]

Prior to the discovery of a third active GDH isoenzyme in roots, it was considered that in most plant species, GDH is encoded by two distinct nuclear genes.[4,9,10] *GDH1* gene encodes a polypeptide subunit termed β and *GDH2* gene encodes α and the polypeptides can be assembled as homo- or heterohexamers composed of different ratios of α and β, leading to the formation of seven active isoenzymes. These seven isoenzymes can be separated by native PAGE (Poly Acrylamide Gel Electrophoresis), followed by in-gel staining for NAD-dependent GDH activity[11,12] or NADH-dependent GDH activity,[13] thus indicating that GDH exhibits a similar anabolic and catabolic activity in vitro. In addition, variations in the GDH isoenzyme pattern were observed

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Submitted: 12/10/12; Revised: 12/18/12; Accepted: 12/18/12
http://dx.doi.org/10.4161/psb.23329
Citation: Fontaine J-X, Tercé-Laforque T, Bouton S, Pageau K, Lea P, Dubois F, Hirel B. Further insights into the isoenzyme composition and activity of glutamate dehydrogenase in Arabidopsis thaliana. Plant Signal Behav 2013; 8: e23329

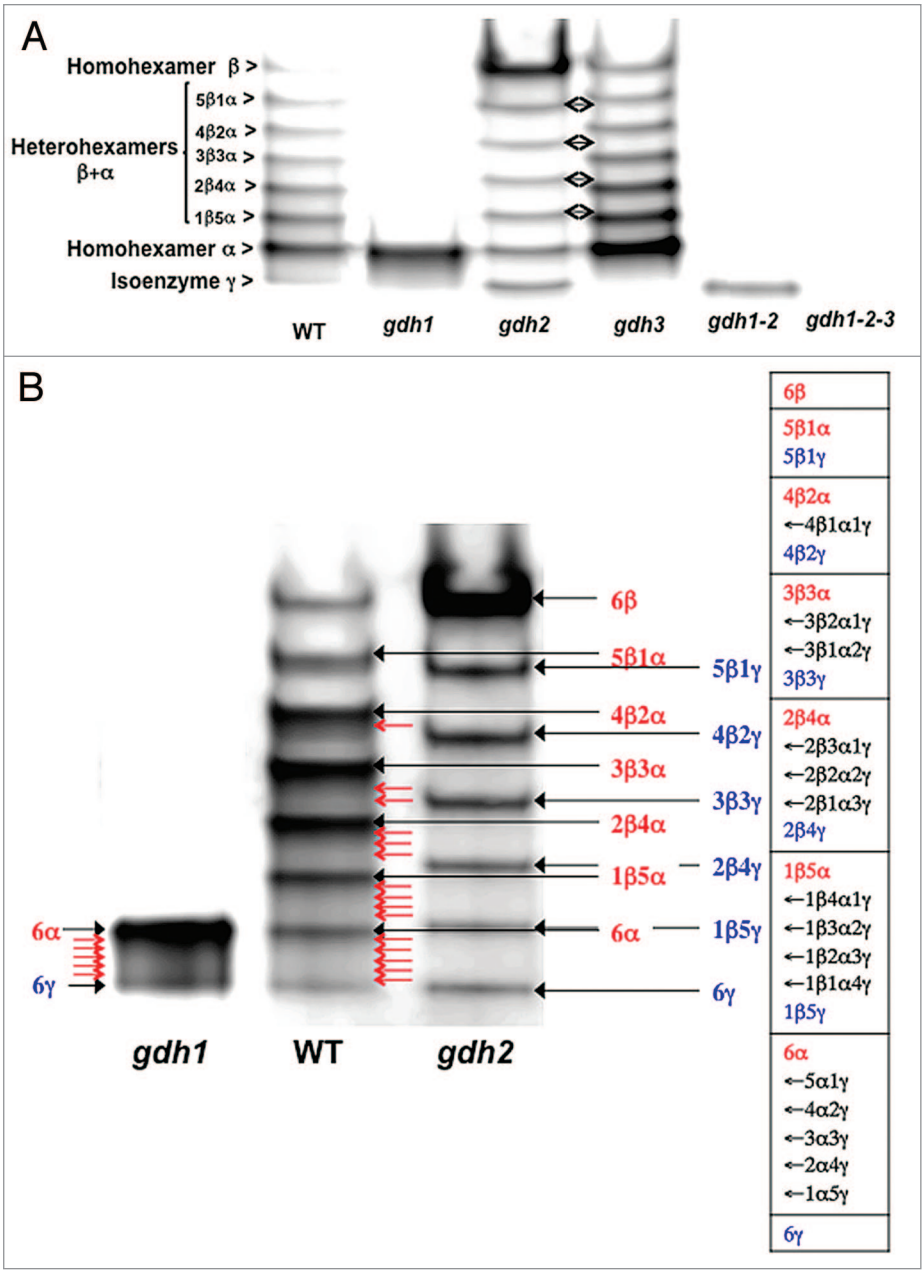

Figure 1. NAD-GDH isoenzyme patterns of roots of *Arabidopsis* wild type and mutants deficient in the three genes encoding the enzyme. (**A**) Protein extracts of the roots of the wild type (WT), *gdh1*, *gdh2*, *gdh3*, *gdh1-2* and *gdh1-2-3* mutants were subjected to native PAGE followed by NAD-GDH in-gel activity staining. The different subunit combinations of the eight isoenzymes detected in the WT are indicated on the left side of the panel. Double arrows indicate the shift observed between the *gdh2* and the *gdh3* mutants for the positions of the different isoenzymes. (**B**) Protein extracts of the roots of the WT, *gdh1* and *gdh2* mutants were subjected to native PAGE followed by NAD-GDH in-gel activity staining. The possible combinations of the three GDH subunits α, β and γ in the different isoenzymes, when they are all present in the WT are shown in the table at the right side of the panel. The subunit composition that predominates in the WT is indicated in red (black closed arrows). The red open arrows in the WT indicate the position of the other subunit composition also listed in the table on the right. The possible different combinations of the subunits in the GDH isoenzymes, when the subunit α or the subunit β is lacking in the *gdh2* and *gdh1* mutants respectively are also shown in blue (black closed arrows) and in the table on the right. The red open arrows in the *gdh1* mutant indicate the position of the five heterohexamer isoenzymes and their composition is presented in the table on the right.

according to the organ examined, or the N source supplied to the plant.[14,15] However, the physiological functions of the seven GDH isoenzymes and the significance of the variations in their relative proportions in roots and shoots, remained unknown.

The Presence of a Third Active NADH-GDH Polypeptide in Roots Leads to the Formation of a Complex Pattern of Isoenzymes

The finding of a third *GDH* gene encoding an isoenzyme termed γ that is only expressed in roots, prompted us to examine if the corresponding polypeptide could be assembled into heterohexamers with the previously indentified α- and β-subunits, which are expressed in both the roots and leaves. We have therefore re-examined the GDH isoenzyme content of the roots and leaves of the WT, the *gdh1*, *gdh2* and *gdh3* single mutants, the *gdh1–2* double mutant and the *gdh1–2-3* triple mutant, by staining for NAD-dependent GDH activity following separation by non-denaturing PAGE. For experimental details describing the production of the mutants and the in-gel staining for GDH activity (see refs. 6, 15 and 16). Following PAGE of extracts of roots of the wild type (WT), a faint eighth band of GDH enzyme activity was visible below the fastest moving, most anodal form of the standard seven GDH isoenzyme band pattern (**Fig. 1A and B**). This additional band of NAD-GDH activity was designated isoenzyme γ.[6] For the *gdh1* mutant, in which only the isoenzyme composed of α homohexamers remained, isoenzyme γ was just visible following PAGE, but at the limit of detection using in-gel activity staining (**Fig. 1A and 1B**). Both in the *gdh2* and in the *gdh1–2* mutant, the activity of isoenzyme γ was much higher compared with the WT (**Fig. 1A and 1B**). Following PAGE of extracts of roots of the *gdh3* mutant, only the seven isoenzymes composed of homo- and heterohexamers of α and β were detected after activity staining,[6,16] (see also **Fig. 2**). As expected, in the *gdh1–2-3* triple mutant, the eight bands of enzyme activity corresponding to the two α and β

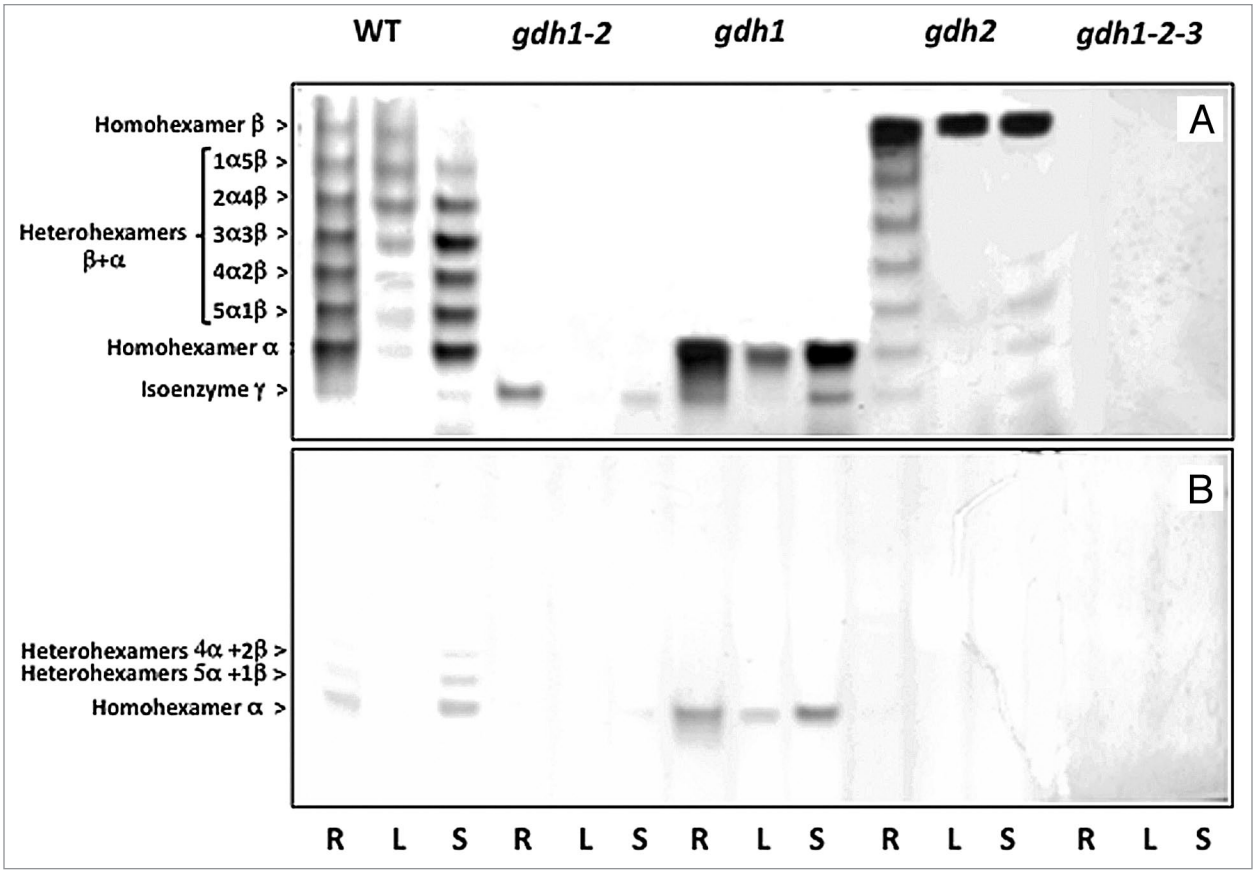

Figure 2. NADH- and NADPH-dependent GDH isoenzyme patterns of roots, leaves and floral stems of *Arabidopsis* WT and mutants deficient in the three genes encoding the enzyme. Protein extracts of the roots (R), leaves (L) and floral stems (S) of the wild type (WT), *gdh1*, *gdh2*, *gdh1-2* and *gdh1-2-3* mutants were subjected to native PAGE followed by NAD-GDH (**A**) and NADP-GDH (**B**) in-gel activity staining. The composition of the subunits of the eight isoenzymes in the WT are indicated on the left side of the panel.

homohexamers, the five heterohexamers composed of different ratios of α and β polypeptides and the isoenzyme γ were not detected. Interestingly for the *gdh2* mutant, seven isoenzymes were detected following PAGE of root extracts, thus suggesting that the β and γ polypeptides could be assembled into five heterohexamers. It is also likely that there were heterohexamers composed of different ratios of α and γ polypeptides both in the WT and the *gdh1* mutant. However, the presence of α and γ heterohexamers could not be clearly distinguished using in-gel GDH activity staining, because the active isoenzymes containing the α and γ polypeptides ran very close to each other following PAGE. Nevertheless a smear of GDH activity, which could indicate the presence of isoenzyme bands, was visible in the most anodal section of the gel following PAGE and activity staining of extracts of the *gdh*1 mutant. This faint staining of anodal bands was also detected following PAGE of WT extracts and taken together suggests that the formation of heterohexamers between the α and γ polypeptides may have occurred (**Fig. 1B**). Lastly, we observed a shift between the position of the seven isoenzyme bands visible in the WT or the *gdh3* mutant and those present in the *gdh2* mutant (**Fig. 1A and B**). This shift can be explained by the formation of heterohexamers between the polypetides α, β and γ in the WT (listed on the right side of **Fig. 1B** and indicated by successive arrows). The 28 possible isoenzyme combinations shown in **Figure 1B** agree with the formula provided by Shaw,[17] which assumes that there is random association of the subunits:

$$i = \frac{(S + P-1)\,!}{P!\,(S-1)\,!}$$

Where i is the number of possible isoenzymes, P is the number of subunits (6) and S is the number of different types of subunits (3).

In the *gdh2* mutant in which the isoenzyme α is not present, the possible formation of heterohexamers composed of different ratios of polypetides β and γ is also visible in **Figure 2A** as well as in **Figure 1B**.

It is also worth stressing that compensatory mechanisms for the isoenzyme composition of the roots appear to be occurring when one of the three isoenzymes is lacking. The in-gel staining for NAD-GDH activity presented in **Figure 1A** shows that compared with the WT, the activity of isoenzyme α was increased in the *gdh1* mutant, whereas in the *gdh2* mutant isoenzyme β was more active. An increase in the apparent activity of isoenzyme γ was also observed in the roots of the *gdh2* and *gdh1–2* mutant. Thus, it can be concluded that the compensatory mechanism, previously observed in the leaves of *gdh1* and *gdh2* mutants in

which the activity of subunit α and β was increased respectively,[16] also occurs in roots. This compensation appears to occur when there is a deficiency in one of the subunits α, β or γ, that have the ability to be assembled into heterohexamers. The physiological significance of such a compensatory mechanism still remains to be elucidated. It may only be due to the fact that an optimal metabolic activity of the enzyme is required, or it may be a consequence of a more complex inter-organ signaling regulatory mechanism circulating via the phloem. Further studies are currently being performed to examine the impact of different combinations of single and double mutations on the metabolic phenotype of roots and shoots and phloem composition.

On the Occurrence of NADPH-Dependent GDH Activity

It is well established that in higher plants, the mitochondrial NAD(H)-dependent enzyme is by far the most active and is of universal occurrence. However in early work, NADP(H)-dependent GDH activity was found to be associated with plastids, although in some cases it was detected in the mitochondria.[18,19] Later on, in a range of higher plants,[12,20,21] including *Arabidopsis*,[22,23] the presence of low NADP-dependent GDH activity has regularly been reported. Since a fourth gene encoding a putative NADPH-GDH, has been identified in *Arabidopsis*[24,25] and rice,[26] it was interesting to determine if NADP(H)-dependent activity could be detected in the different *gdh* mutants.

When the GDH isoenzyme content of extracts of different organs was examined in the WT following PAGE and staining for NAD-dependent GDH activity, eight bands were visible both in the roots and floral stems. However, following PAGE of leaf extracts, the standard seven bands isoenzyme pattern was observed. Although at the limit of detection following in-gel enzyme activity staining, we found that isoenzyme γ was also active in the floral stems. As previously observed in *Arabidopsis*,[15,16] the most cathodal isoenzymes composed of homohexamers of β were more active in leaves, whereas the most anodal isoenzymes composed of homohexamer α were more active in both roots and floral stems (**Fig. 2**). In leaves of the *gdh1* mutant only the homohexamer composed of six α-subunits was detected. Compared with the WT, isoenzyme γ was more active in both the roots and stems of the *gdh-1* mutant, following PAGE and in-gel activity staining. Only the homohexamer composed of six β-subunits was detected in the leaves of the *gdh2*-deficient mutant, whereas in both roots and stems, heterohexamers between the β and γ subunits were still present (see above). Following PAGE of an extract of the *gdh1-2-3* mutant, no band of NAD-GDH enzyme activity was visible, whichever organ was examined (**Fig. 2**).

Interestingly, there would appear to be a compensatory mechanism for the activity of isoenzyme γ both in the *gdh1*, and in the *gdh1-2* and to a lower extent in the *gdh2* mutant mutants in comparison to the WT. As preciously observed,[6,16] similar compensation mechanisms can be seen for homohexamer α and homohexamer β in *gdh1* and *gdh2* mutants respectively. Although the physiological significance of such mechanisms remains unresolved, one can hypothesize that the plant compensates for the lack of one of the three isoenzymes by overexpresing the remaining ones. This also suggests that there are metabolic signals controlling such regulatory mechanisms, thus highlighting the importance of the enzyme at the interface between carbon and nitrogen metabolism in both roots and shoots.

When NAD was replaced by NADP following PAGE, in-gel GDH activity staining revealed that in the WT only the bands composed of the α homohexamer and 5α-1β and 4α-2β heterohexamers exhibited very faint enzyme activity. Similarly in the *gdh1* mutant, the remaining α homohexamer also exhibited NADP-dependent enzyme activity. Therefore, it can be concluded that only the α isoenzyme is able to use NADP as a cofactor. No additional band of NADP-dependent GDH activity could be detected following PAGE and in-gel staining of extracts of roots, leaves or stems. In particular, it can be seen that even in the GDH triple mutant *gdh1-2-3*, that is totally deficient of NAD-GDH activity, there is no evidence of any NADP dependent activity. Therefore, it would appear that the gene encoding a putative NADP(H)-GDH is not expressed as an active protein in *Arabidopsis* and that the NADP(H)-dependent enzyme activity detected by some authors may be due to the action of the α subunit of the NAD(H)-dependent GDH2 isoenzyme.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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